

BRANCHIAL CO₂ RECEPTORS AND CARDIORESPIRATORY ADJUSTMENTS DURING HYPERCARBIA IN PACIFIC SPINY DOGFISH (*SQUALUS ACANTHIAS*)

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Summary

Adult Pacific spiny dogfish (*Squalus acanthias*) were exposed to acute (approximately 20 min) hypercarbia while we monitored arterial blood pressure, systemic vascular resistance (R_S), cardiac output (\dot{V}_b) and frequency (f_H) as well as ventilatory amplitude (V_{AMP}) and frequency (f_V). Separate series of experiments were conducted on control, atropinized (100 nmol kg⁻¹) and branchially denervated fish to investigate putative CO₂-chemoreceptive sites on the gills and their link to the autonomic nervous system and cardiorespiratory reflexes.

In untreated fish, moderate hypercarbia (water CO₂ partial pressure; $P_{wCO_2}=6.4\pm 0.1$ mmHg) (1 mmHg=0.133 kPa) elicited significant increases in V_{AMP} (of approximately 92%) and f_V (of approximately 18%) as well as decreases in f_H (of approximately 64%), \dot{V}_b (approximately 29%) and arterial blood pressure (of approximately 11%); R_S did not change significantly.

Denervation of the branchial branches of cranial nerves IX and X to the pseudobranch and each gill arch eliminated all cardiorespiratory responses to hypercarbia. Prior

administration of the muscarinic receptor antagonist atropine also abolished the hypercarbia-induced ventilatory responses and virtually eliminated all CO₂-elicited cardiovascular adjustments. Although the atropinized dogfish displayed a hypercarbic bradycardia, the magnitude of the response was significantly attenuated (36±6% decrease in f_H in controls versus 9±2% decrease in atropinized fish; means ± S.E.M.).

Thus, the results of the present study reveal the presence of gill CO₂ chemoreceptors in dogfish that are linked to numerous cardiorespiratory reflexes. In addition, because all cardiorespiratory responses to hypercarbia were abolished or attenuated by atropine, the CO₂ chemoreception process and/or one or more downstream elements probably involve cholinergic (muscarinic) neurotransmission.

Key words: *Squalus acanthias*, Pacific spiny dogfish, hypercapnia, systemic resistance, carbon dioxide, chemoreceptor, pH, teleost, hypercarbia, elasmobranch, bradycardia, atropine.

Introduction

The cardiorespiratory responses of fish to environmental hypoxia have been studied extensively (for reviews, see Randall, 1982; Shelton et al., 1986; Smatresk, 1990; Bursleson et al., 1992; Fritsche and Nilsson, 1993; Milsom et al., 1999). Although there is considerable inter-specific variation (see, for example, Fritsche, 1990), teleosts typically respond to hypoxia with hyperventilation, bradycardia and hypertension (caused by increased systemic vascular resistance). Elasmobranchs also exhibit hyperventilation and bradycardia (for a review, see Butler and Metcalfe, 1988) but, in contrast to most teleosts, their blood pressure is decreased (Butler and Taylor, 1971; Short et al., 1979) during hypoxia. The mechanisms underlying the cardiorespiratory reflex responses to hypoxia are reasonably well understood and are initiated by branchial chemoreceptors with both an internal and external orientation. The external receptors are thought to trigger the cardiovascular and ventilatory responses, whereas the internal receptors are believed to be involved

solely in mediating the ventilatory responses (Bursleson et al., 1992).

Considerably less is known about the cardiorespiratory responses of fish to elevated environmental levels of CO₂ (hypercarbia), and few studies have addressed the underlying mechanisms. Although hyperventilatory responses of fish to hypercarbia are well-documented, the precise cause and the physiological significance of this response have been debated (see Perry and Wood, 1989). Because of the pronounced role of blood O₂ status in setting respiratory drive in fish (Randall, 1982; Smith and Jones, 1982), it has proved difficult to distinguish between the primary effects of CO₂/H⁺ and the indirect effects caused by acidosis-induced hypoxaemia (via the Root effect). In recent years, however, there have been compelling arguments made for direct effects of CO₂/H⁺ on ventilation (Heisler et al., 1988; Kinkead and Perry, 1991; Perry and Gilmour, 1996; Bursleson and Smatresk, 2000), and current models of respiratory control in fish thus incorporate a

specific, albeit secondary, role for CO_2/H^+ (Milsom, 1995a; Milsom, 1995b).

Although evidence of hypercarbic bradycardia was presented as early as 1978 (Kent and Peirce, 1978), only recently have the cardiovascular effects of hypercarbia been addressed in any detail (Perry et al., 1999a; McKendry and Perry, 2001; Sundin et al., 2000; Reid et al., 2000). In rainbow trout (*Oncorhynchus mykiss*), the cardiovascular responses to hypercarbia (principally bradycardia, lowered cardiac output and elevated systemic resistance) were shown to be reflexively mediated by the autonomic nervous system (Perry et al., 1999a), were not related to hypoxaemia (Perry et al., 1999a) and were probably initiated by externally oriented CO_2/H^+ receptors (McKendry and Perry, 2001). The importance of gill receptors in mediating hypercarbic bradycardia was further demonstrated in two tropical teleosts, the tambaqui (*Colossoma macropomum*; Sundin et al., 2000) and the traira (*Hoplias malabaricus*; Reid et al., 2000).

Despite the robust ventilatory and cardiovascular responses to hypercarbia in dogfish (*Squalus acanthias*), nothing is known about the location of the sensory receptors in this species. Because it lacks a Root effect, the dogfish is a particularly useful model species to study aspects of CO_2 -induced cardiorespiratory reflexes in fish. Unlike the situation in most previous studies (Sundin et al., 2000; Reid et al., 2000), the effects of external and/or internal changes in CO_2/H^+ can be examined without the need to use hyperoxia as a tool to prevent hypoxaemia (Burlinson and Smatresk, 2000).

Given this, the present study tests the hypothesis that the cardiorespiratory responses to hypercarbia in dogfish are triggered by branchial chemoreceptors. This was achieved by comparing physiological responses in intact, in sham-operated and in gill-denervated animals. Complete gill denervation was accomplished *via* surgical transection of the IXth cranial (glossopharyngeal) nerve and the branchial branches of the Xth cranial (vagus) nerve. Because of the possible role of cholinergic neurotransmission in O_2 chemoreception (Burlinson and Milsom, 1995a) and the importance of increased vagal activity in mediating bradycardia, experiments were also performed in fish pre-treated with atropine, a muscarinic receptor antagonist.

Materials and methods

Experimental animals

Pacific spiny dogfish *Squalus acanthias* (L.) were caught by hook and line or collected by net during trawls by local fishermen and held indoors under natural photoperiod at the Bamfield Marine Station in a 75 000 l opaque circular tank. Dogfish were fed twice weekly with herring.

Surgical procedures

Fish weighing between 1250 and 3460 g (experimental $N=28$) were anaesthetized using benzocaine (0.1 g l^{-1} ethyl-*p*-aminobenzoate; Sigma) in sea water until breathing movements stopped. The fish were then placed onto an

operating table where their gills were force-ventilated with oxygenated anaesthetic solution. Bidirectional cannulation of the coeliac artery was required to establish an extracorporeal arterial blood circulation (see below). After making a ventral incision and externalising much of the viscera, the coeliac artery was cannulated in the orthograde and retrograde directions using polyethylene tubing (Clay Adams; PE 50). The viscera were re-inserted, the wound sutured, and the cannulae were secured firmly to the ventral musculature. A lateral incision was made in the caudal peduncle to expose and cannulate (PE 50) both the caudal vein and the caudal artery in the anterograde direction (Axelsson and Fritsche, 1994). While the arterial cannula allowed arterial blood pressure (P_a) measurements, the caudal vein cannula permitted injections and/or repeated blood sampling. To record ventilation, a heat-flared cannula (PE 160) was inserted into one spiracular cavity and secured using a single silk ligature and Vetbond.

Animals were fitted with a cardiac flow (\dot{V}_b) probe after all cannulae had been secured. A small ventral incision was made to expose the pericardial cavity, and the pericardium was dissected to expose the conus arteriosus. A 3S or 4S ultrasonic flow probe (Transonics Systems Inc., Ithaca, NY, USA) was placed non-occlusively around the conus to enable the measurement of cardiac output (Olson et al., 1997). Lubricating jelly was used with the perivascular flow probe as an acoustic couplant. The incision was sutured closed and sealed with Vetbond, and the flow probe cable was secured externally to the ventral surface of the fish using silk ligatures and Vetbond.

Additional surgery was required for the denervation series. A small incision (approximately 3 cm) was made in the caudal direction, beginning approximately 2 cm behind the spiracular opening. This incision permitted access to cranial nerves IX (glossopharyngeal) and X (vagus), including all branchial branches from the vagus (X) to each gill arch. The glossopharyngeal (IX) and the branchial branches of the vagus (X) nerve, leading to each gill arch, were delicately dissected free of surrounding connective tissue and severed with micro-scissors. In all cases, the cardiac and visceral branches of the vagus were left intact. All denervations were confirmed *post mortem* by autopsy. In the sham-operated group, dogfish underwent an identical procedure, but without the actual denervation, i.e. the nerves were exposed but not sectioned.

All cardiovascular cannulae were filled with heparinised (50 i.u. ml^{-1} sodium heparin) saline (500 mmol l^{-1} NaCl). After surgery, fish were revived, placed into opaque flow-through acrylic boxes and left to recover for approximately 24 h prior to experimentation.

Experimental protocol

Measurement of water/blood respiratory variables

Inspired water was withdrawn continuously *via* a peristaltic pump and passed over P_{O_2} and P_{CO_2} electrodes (Radiometer) housed in temperature-controlled cuvettes and connected to a Radiometer blood gas analyzer. Blood gas levels were assessed

using an extracorporeal arterial blood shunt (Thomas, 1994; Perry and Gilmour, 1996) that permitted continuous and simultaneous measurements of arterial blood pH (pHa), oxygen partial pressure (P_{aO_2}) and carbon dioxide partial pressure (P_{aCO_2}). This was achieved by pumping (peristaltic pump; 0.4 ml min⁻¹) blood from the coeliac artery through pH, P_{CO_2} and P_{O_2} electrodes connected in series and returning it to the coeliac return cannula. The extracorporeal shunt contained approximately 1.0 ml of blood, representing less than 3% of the total blood volume. Ventilatory and cardiovascular variables were recorded concurrently (see below).

Prior to experimentation, the extracorporeal shunt was rinsed for 20–30 min with a solution of ammonium heparin (540 i.u. ml⁻¹) to prevent blood from clotting in the tubing and electrode chambers. After starting the extracorporeal circulation, a period of approximately 10–15 min was required to achieve stable baseline recordings of pHa, P_{aCO_2} and P_{aO_2} . All electrodes were calibrated prior to each individual experiment (Perry et al., 1999b).

Measurement of cardiovascular and ventilation variables

Ventilatory amplitude, V_{AMP} , was assessed by monitoring pressure changes in the spiracle associated with each breathing cycle. The spiracle catheter was filled with sea water, connected to a pressure transducer (Bell and Howell) and linked to an amplifier (Harvard Biopac DA 100). The pressure transducer was calibrated daily against a static column of water.

The caudal artery cannula was flushed with heparinised saline (50 i.u. ml⁻¹) to prevent clotting and then connected to a pressure transducer (Bell and Howell) that had been pre-calibrated against a static column of water. Analog blood pressure signals were measured using Harvard Biopac amplifiers (DA 100). Cardiac output, \dot{V}_b , was determined by attaching the ultrasonic flow probe to a Transonic T106 single-channel blood flow meter.

All analog signals (gas levels, blood and ventilation pressures and \dot{V}_b) were converted to digital data by interfacing with a data-acquisition system (Biopac Systems Inc.) using Acknowledge data-acquisition software (sampling rate set at 40 s⁻¹) and a Pentium PC. Thus, continuous data recordings were obtained for mass-specific \dot{V}_b , cardiac frequency (f_H ;

automatic rate calculation from the pulsatile \dot{V}_b trace), cardiac stroke volume (V_S ; \dot{V}_b/f_H), ventilation frequency (f_V ; automatic rate calculation from the raw ventilation pressure traces), V_{AMP} (the difference between maximum and minimum ventilation pressures), mean blood pressures and systemic vascular resistance (R_S ; mean P_a/\dot{V}_b).

Cardiorespiratory effects of external hypercarbia

Experiments commenced on intact, sham-operated or gill-denervated fish with a 10 min recording period under conditions of normoxic normocarbica. After this 'pre' period, the water was rendered hypercarbic for 20 min by gassing a water equilibration column with 1.5% CO₂ in air (Cameron flowmeter; model GF-3/MP; Cameron Instruments Inc.), then switched back to normocarbica water. To test the efficacy of the gill denervation, 0.5 ml of sodium cyanide (0.5 mg ml⁻¹) was injected as a bolus into the mouth of fish, and ventilation was monitored.

A separate group of fish were administered a bolus injection (via the caudal vein) of the muscarinic receptor antagonist atropine (100 nmol kg⁻¹) dissolved in saline (100 nmol ml⁻¹). These fish were given at least 15 min to allow the atropine to circulate and take effect before undergoing the same procedure described above for the non-atropinized group. To determine the efficacy of the atropine-derived vagal blockade of the heart, fish were administered an intravenous injection (1 ml kg⁻¹) of the potent muscarinic receptor agonist methacholine (100 nmol kg⁻¹).

Statistical analyses

Data are presented as mean values \pm one standard error of the mean (S.E.M.). For all experiments, the statistical significance of the observed effects was determined using paired *t*-tests, while differences between group means employed unpaired *t*-tests. One-way analysis of variance (ANOVA) followed, where necessary, by Dunnett's all-pairwise *post-hoc* comparison was used across treatments in Table 1. All statistical analyses were performed using SigmaStat 2.0 commercial software (SPSS Inc.) with a fiducial limit of 5%. All figures were plotted using the Sigmaplot 4.0 commercial graphics software package (SPSS Inc.).

Table 1. The effects of hypercarbia on water/blood gas levels of Pacific spiny dogfish (*Squalus acanthias*)

	Normocarbica				Hypercarbia			
	Control	Atropine	Sham	Denervated	Control	Atropine	Sham	Denervated
P_{wO_2} (mmHg)	147.0 \pm 1.0	137.7 \pm 1.7	146.7 \pm 3.3	141.2 \pm 2.5	147.3 \pm 0.9	139.3 \pm 0.9	147.6 \pm 2.4	142.9 \pm 2.7
P_{wCO_2} (mmHg)	0.74 \pm 0.45	0.62 \pm 0.06	0.66 \pm 0.21	0.63 \pm 0.09	6.38 \pm 0.13*	5.94 \pm 0.28*	6.68 \pm 0.34*	5.99 \pm 0.54*
P_{aO_2} (mmHg)	120.9 \pm 2.2	117.8 \pm 6.8	112.7 \pm 8.6	52.9 \pm 17.3	128.8 \pm 2.0*	125.0 \pm 9.1	115.1 \pm 11.0	84.8 \pm 19.1
P_{aCO_2} (mmHg)	1.20 \pm 0.10	2.21 \pm 0.20	1.93 \pm 0.48	3.46 \pm 0.38	6.83 \pm 0.24*	6.29 \pm 0.57*	6.87 \pm 0.29*	6.61 \pm 0.86*
pHa	7.71 \pm 0.04	7.59 \pm 0.03	7.92 \pm 0.31	7.48 \pm 0.04	7.41 \pm 0.04*	7.33 \pm 0.03*	7.60 \pm 0.36*	7.29 \pm 0.04*

Values are means \pm S.E.M.: $N=7-9$ for control; $N=5-7$ for atropine-treated fish (100 nmol kg⁻¹); $N=4-7$ for sham-operated fish and $N=4-5$ for denervated fish (branchial branches of cranial nerves IX and X surgically transected).

An asterisk denotes a statistically significant difference between the normocarbica and hypercarbic value within each treatment; a double dagger denotes a significant difference between the designated treatment and all other treatments at a given P_{wCO_2} ($P<0.05$).

1 mmHg=0.133 kPa.

Results

Cardiorespiratory effects of hypercarbia

The interactive effects of the various treatments (i.e. denervation, atropine) and acute hypercarbia on water/blood gas levels are depicted in Table 1. During normocarbia, blood gas levels were similar in the control, sham-operated and atropine-treated fish, whereas the denervated fish displayed a significant reduction in P_{aO_2} . The denervated fish also appeared to exhibit an elevated P_{aCO_2} and reduced pH_a; however, the differences were not statistically significant. In all treatment groups, P_{wCO_2} was increased to approximately 6 mmHg during hypercarbia, resulting in pronounced respiratory acidosis (lowered pH_a as a result of increased P_{aCO_2}) of more-or-less equivalent magnitude (Table 1). Although P_{aO_2} appeared to increase in all treatment groups during hypercarbia, the change was statistically significant only in the control fish.

Control and sham-operated fish displayed marked increases in both breathing frequency (f_V) and amplitude (V_{AMP}) during hypercarbia (Fig. 1). Denervating the gills *via* surgical transection of the branchial branches of cranial nerves IX and X eliminated these hyperventilatory responses (Fig. 1). It is important to point out that denervation itself, while without effect on f_V , caused a marked reduction in V_{AMP} ($P < 0.05$) (Fig. 1).

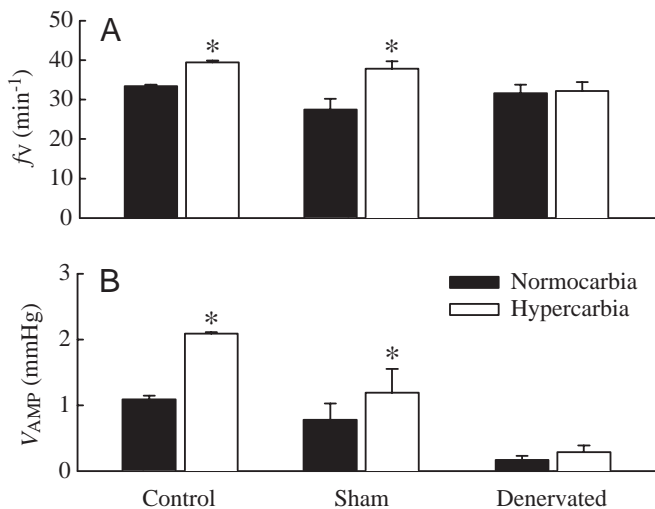


Fig. 1. The effects of external hypercarbia (P_{wCO_2} ; control, 6.38 ± 0.13 mmHg; sham-operated, 6.58 ± 0.44 mmHg; denervated, 5.99 ± 0.54 mmHg) on (A) ventilation frequency (f_V ; $N=5-8$) and (B) ventilation amplitude (V_{AMP} ; $N=4-8$). Filled columns represent pre-hypercarbic (i.e. normocarbia) values; open columns represent maximal responses obtained during hypercarbia. Control fish were untreated, sham-operated fish underwent an identical procedure to the denervated fish except for the actual transection of cranial nerves IX and X, and denervated dogfish underwent surgical transection of all branchial branches of cranial nerves IX and X. Values are means \pm 1 S.E.M. An asterisk denotes a significant difference between normocarbia and hypercarbia ($P < 0.05$). 1 mmHg = 0.133 kPa.

Unlike the effects on V_{AMP} , none of the measured or calculated cardiovascular variables was affected by denervation alone (Fig. 2). Hypercarbia elicited a statistically significant decrease in f_H and \dot{V}_b within the untreated and sham-operated groups, whereas these variables remained unchanged in the denervated group (Fig. 2A,B). Although arterial blood pressure (P_a) decreased (by approximately 11%) in the control group, there was no significant effect of hypercarbia on P_a in the sham-operated or denervated dogfish

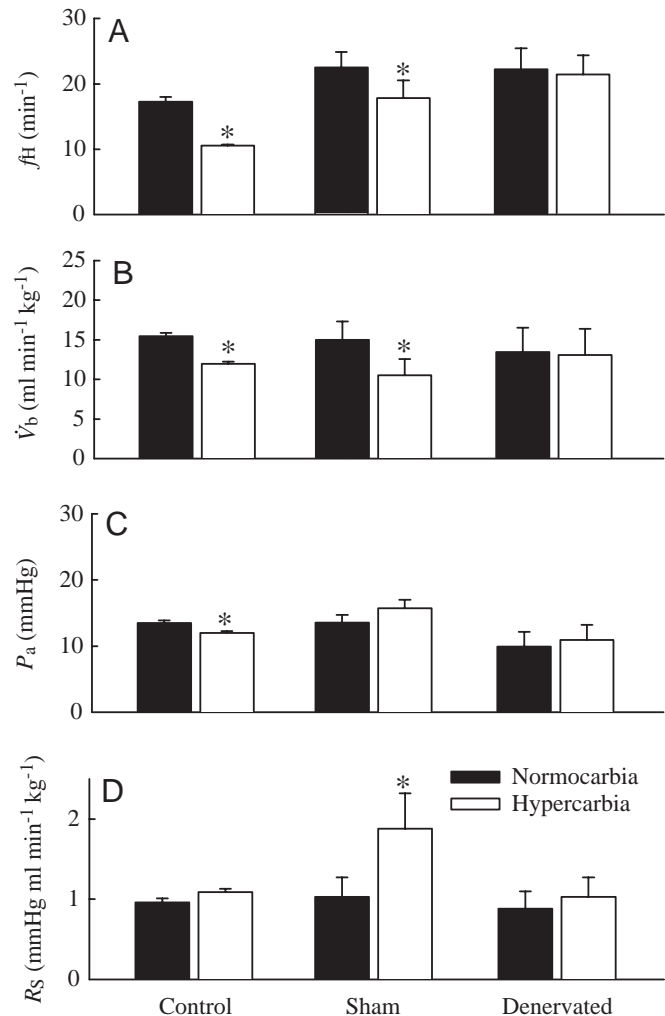


Fig. 2. The effects of external hypercarbia (P_{wCO_2} ; control, 6.38 ± 0.13 mmHg; sham-operated, 6.58 ± 0.44 mmHg; denervated, 5.99 ± 0.54 mmHg) on cardiovascular variables in the Pacific spiny dogfish (*Squalus acanthias*): (A) heart rate (f_H ; $N=5-9$), (B) cardiac output (\dot{V}_b ; $N=5-10$), (C) arterial blood pressure (P_a ; $N=5-9$) and (D) systemic vascular resistance (R_S ; $N=5-9$). Filled columns represent pre-hypercarbic (i.e. normocarbia) values; open columns represent maximal responses obtained during hypercarbia. Control fish were untreated, sham-operated fish underwent an identical procedure to the denervated fish except for the actual transection of cranial nerves IX and X, and denervated dogfish underwent surgical transection of all branchial branches of cranial nerves IX and X. Values are means \pm 1 S.E.M. An asterisk denotes a significant difference between normocarbia and hypercarbia ($P < 0.05$). 1 mmHg = 0.133 kPa.

(Fig. 2C). Furthermore, despite fractional increases in systemic vascular resistance (R_S), only the sham-operated group demonstrated a significant increase in R_S during hypercarbia (Fig. 2D).

Fig. 3 clearly shows the inhibitory effect of the muscarinic receptor antagonist atropine (100 nmol kg⁻¹) on a variety of cardiorespiratory variables in dogfish experiencing an acute bout of hypercarbia ($P_{wCO_2}=5.94\pm 0.28$ mmHg). Atropine eliminated the hypercarbia-induced ventilatory response (Fig. 3A,B) and virtually abolished all cardiovascular adjustments (Fig. 3C–F). Although the atropinized dogfish still displayed a significant bradycardia, the magnitude of the response was greatly diminished. For example, the decrease in f_H of $35.9\pm 5.6\%$ for the control group was significantly larger than the decrease in f_H of $8.5\pm 2.0\%$ for the atropine-treated group.

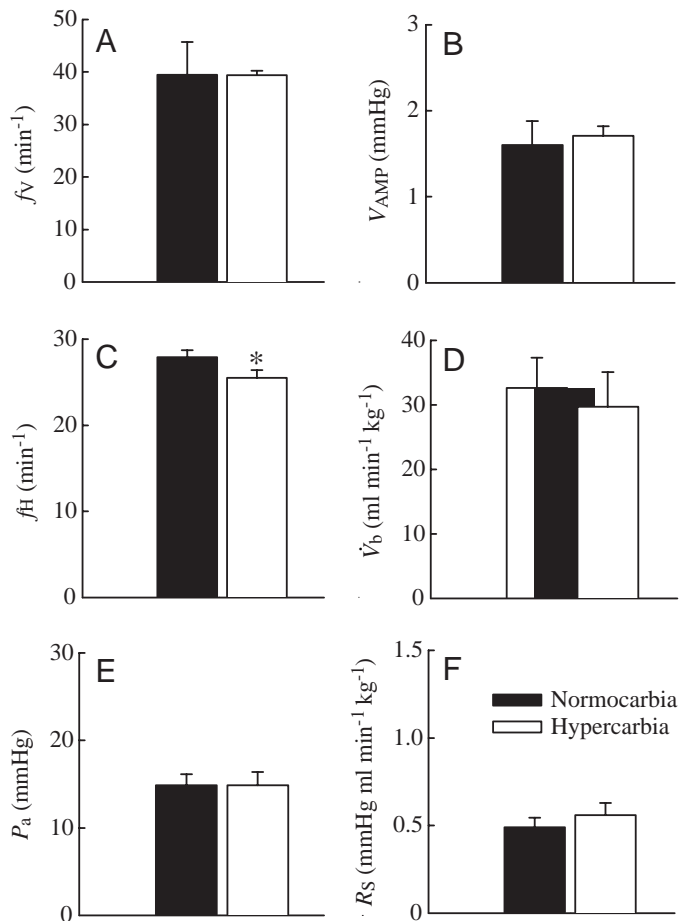


Fig. 3. The effects of external hypercarbia ($P_{wCO_2}=5.94\pm 0.28$) on cardiovascular variables in atropine-treated (100 nmol kg⁻¹) Pacific spiny dogfish (*Squalus acanthias*): (A) ventilation frequency (f_v ; $N=7$), (B) ventilation amplitude (V_{AMP} ; $N=7$), (C) heart rate (f_H ; $N=7$), (D) cardiac output (\dot{V}_b ; $N=7$), (E) arterial blood pressure (P_a ; $N=7$) and (F) systemic vascular resistance (R_S ; $N=7$). Filled columns represent pre-hypercarbic (i.e. normocarbic) values; open columns represent maximal responses obtained during hypercarbia. Values are means \pm 1 S.E.M. An asterisk denotes a significant difference between normocarbic and hypercarbic ($P<0.05$). 1 mmHg=0.133 kPa.

Effectiveness of the denervation in blocking O₂ chemoreceptor-mediated responses

The effectiveness of the branchial denervation was assessed by comparing the ventilatory responses of fish to a bolus injection into the mouth ('external' injection) of the O₂ chemoreceptor stimulant sodium cyanide. The control and sham-treated fish exhibited marked hyperventilatory responses to cyanide (Fig. 4). The cyanide-induced increase in f_v was eliminated by branchial denervation and, although V_{AMP} still increased significantly, the magnitude of the response was drastically diminished. Interestingly, the hyperventilatory responses to external cyanide were also eliminated in the atropine-treated fish (Fig. 4).

External injection of cyanide caused a significant bradycardia (20% decrease in f_H from 14.6 ± 1.2 to 11.7 ± 0.5 min⁻¹) in control fish and lowering of P_a (15% decrease from 13.4 ± 1.7 to 11.4 ± 1.8 mmHg) without affecting \dot{V}_b or R_S (data not shown). The cyanide-induced bradycardia and hypotension were eliminated in denervated or atropinized fish (data not shown).

Efficacy of atropine in establishing cardiac muscarinic blockade

Fig. 5 illustrates the efficacy of atropine in achieving complete muscarinic receptor blockade. First, atropine itself caused an approximate doubling of f_H . Second, the decrease in f_H (of approximately 55%) after an intravenous injection of the muscarinic receptor agonist methacholine was eliminated in the atropinized fish.

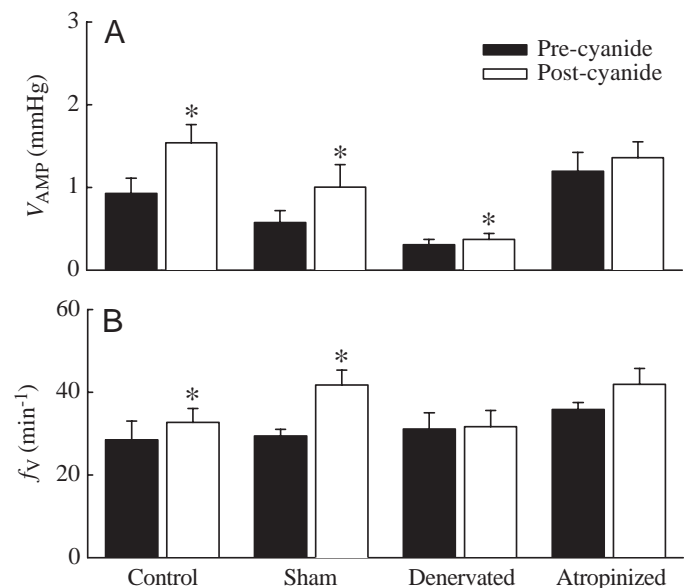


Fig. 4. The effects of a bolus injection into the mouth of 0.5 ml of sodium cyanide (0.5 mg ml⁻¹) on (A) ventilation amplitude (V_{AMP}) and (B) ventilation frequency (f_v) in control, sham-operated, denervated or atropinized dogfish (*Squalus acanthias*). Filled columns represent pre-cyanide values; open columns represent maximal responses obtained after cyanide injection. Values are means \pm 1 S.E.M., $N=4-7$. An asterisk denotes a significant difference from the pre-cyanide value ($P<0.05$). 1 mmHg=0.133 kPa.

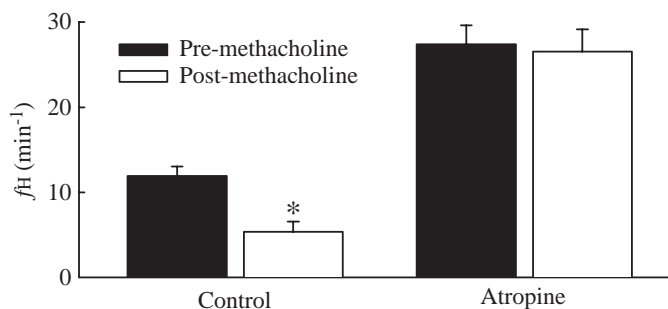


Fig. 5. The effectiveness of the muscarinic receptor antagonist atropine (100 nmol kg^{-1}) in blocking a methacholine-induced (100 nmol kg^{-1}) reduction in heart rate (f_H) in Pacific spiny dogfish (*Squalus acanthias*). Filled columns represent pre-methacholine (i.e. resting) values; open columns represent maximal responses obtained following intravenous injection of methacholine. Values are means ± 1 S.E.M., $N=6$. An asterisk denotes a significant difference between pre-methacholine f_H and post-methacholine f_H ($P < 0.05$).

Discussion

Although previous studies have addressed aspects of ventilatory control during hypercarbia in elasmobranchs (Randall et al., 1976; Heisler et al., 1988; Graham et al., 1990; Perry and Gilmour, 1996), this is the first study to examine in detail both the ventilatory and cardiovascular responses to hypercarbia and also the first to attempt to localise the receptors that mediate the cardiorespiratory responses. Lacking a Root effect, elasmobranchs are ideal for such studies because, unlike the situation in teleosts (Burlerson and Smatresk, 2000; Sundin et al., 2000; Reid et al., 2000), the results are not confounded by indirect effects of CO_2 -induced hypoxaemia. Two approaches were used in this study to assess cardiorespiratory control during hypercarbia. In the first, complete branchial denervation was used as a tool to eliminate sensory input from the gill arches and the pharynx and, in the second, atropine was used to block muscarinic receptors to examine the involvement of cholinergic nervous transmission in the cardiorespiratory responses.

Critique of the methods

Because of the diffuse branchial location of oxygen chemoreceptors in elasmobranchs (Taylor, 1992), denervation of all gill arches as well as the orobranchial cavity was required to eliminate the reflex hypoxic bradycardia (Butler et al., 1977). Assuming a similar distribution of the presumptive CO_2/H^+ receptors, we also chose to denervate all gill arches by bilateral sectioning of cranial nerves IX and X. As previously demonstrated for *Scyliorhinus canicula* (Butler et al., 1977), this led to a reduction in PaO_2 (from 121 to 53 mmHg) that was probably related to the markedly reduced breathing amplitude. Although not statistically significant, the trend towards respiratory acidosis in denervated dogfish was also consistent with impaired gas transfer. However, because of the absence of a Root effect in dogfish coupled with the relatively high affinity of its haemoglobin for oxygen ($P_{50}=13.5\text{--}17.3$ mmHg

in *S. acanthias*; Butler and Metcalfe, 1988) and small Bohr effect (Butler and Metcalfe, 1988), denervation probably still had only minor effects on overall blood O_2 transport. Moreover, aside from V_{AMP} and PaO_2 , none of the other measured cardiorespiratory variables was significantly affected by denervation. The marked reduction in V_{AMP} after denervation suggests that the ability to increase ventilatory amplitude during hypercarbia may have been compromised in these fish. Thus, the effects of denervation on the V_{AMP} response to hypercarbia must be interpreted with caution. Fortunately, similar concerns need not apply to the remaining cardiorespiratory variables examined.

The deleterious effects of gill denervation on gas transfer are not confined to dogfish but have also been reported in the bowfin *Amia calva* (McKenzie et al., 1991) and the channel catfish *Ictalurus punctatus* (Burlerson and Smatresk, 2000). However, most studies employing gill denervation as a tool to localise chemoreceptors in fish have not measured arterial blood gas levels (e.g. Sundin et al., 2000; Reid et al., 2000). It seems likely, however, that impaired gas transfer is a universal response to complete gill denervation.

The effects of hypercarbia in intact dogfish

As previously documented (Perry and Gilmour, 1996), spiny dogfish exhibited a pronounced hyperventilatory response during hypercarbia consisting of increases in both f_V and V_{AMP} . As reported for a variety of elasmobranch (Randall et al., 1976; Graham et al., 1990; Perry and Gilmour, 1996) and teleost (Thomas, 1983; Perry and Gilmour, 1996; Reid et al., 2000) species, the increase in V_{AMP} (92%) was considerably greater than the increase in f_V (18%). Comparing all the fish species that have been examined, however, no clear pattern of ventilatory response emerges. Indeed, some species display a predominant (channel catfish; Burlerson and Smatresk, 2000) or exclusive (tambaqui; Sundin et al., 2000) V_{AMP} response to hypercarbia.

Few studies have examined the cardiovascular effects of hypercarbia in fish and, to our knowledge, this is only the second investigation to report the effects in any elasmobranch species. Moreover, the single previous study, on dogfish (*Squalus acanthias*) (Kent and Peirce, 1978), was performed on anaesthetised, restrained and force-ventilated animals. With so few data, it is difficult to construct a generic model describing the cardiovascular effects of CO_2 in fish. In rainbow trout (*Oncorhynchus mykiss*), hypercarbia elicits a reflex bradycardia and an elevation of arterial blood pressure (Perry et al., 1999a). The hypertensive response to hypercarbia in trout reflects a profound increase in systemic vascular resistance caused by vasoconstriction following sympathetic activation of vascular smooth muscle α -adrenoreceptors (Perry et al., 1999a). This reflex vasoconstriction serves to counteract the specific dilatory effects of elevated CO_2 on the systemic vasculature (McKendry and Perry, 2001). The receptors mediating the reflex cardiovascular responses to hypercarbia in trout appear to be externally oriented because increases in PaCO_2 alone are without effect on cardiac frequency or

systemic resistance (McKendry and Perry, 2001). Results obtained using other species have yielded conflicting results. For example, while two neotropical teleost species (tambaqui and traira) both exhibit CO₂-evoked bradycardia, the tambaqui (*Colossoma macropomum*) responds with hypertension (Sundin et al., 2000), whereas the traira (*Hoplias malabaricus*) exhibits mild hypotension (Reid et al., 2000); in neither study was systemic resistance determined. Finally, although channel catfish (*Ictalurus punctatus*) display a pronounced hyperventilatory response to hypercarbia, blood pressure and cardiac frequency are unaffected (Burlison and Smatresk, 2000).

In the present study, dogfish displayed a bradycardia, reduced cardiac output and lowered arterial (post-branchial) blood pressure in response to acute hypercarbia. Because systemic vascular resistance was unaltered, the hypotension was probably caused by the lowering of cardiac output although, on the basis of the study of Kent and Peirce (Kent and Peirce, 1978), the possibility of increased gill vascular resistance contributing to the overall hypotension cannot be excluded. The hypotensive response of dogfish to moderate hypercarbia ($P_{wCO_2}=6.4$ mmHg) in the present study was similar to the slight (8%) decrease in P_a seen during exposure to much higher levels of P_{wCO_2} (approximately 37.5 mmHg) in this same species (Kent and Peirce, 1978). Interestingly, elasmobranchs, unlike most teleosts, also exhibit a hypotensive response during exposure to environmental hypoxia (Satchell, 1961; Butler and Taylor, 1971). Thus, the cardiovascular (and indeed the ventilatory) responses of dogfish to hypercarbia are more-or-less identical to those previously reported for hypoxia. This raises the question of whether the physiological responses to hypercarbia and hypoxia are mediated by a single class of receptor or by more than one class of receptor linked to similar efferent pathways (see below).

CO₂ versus H⁺ receptors

In the present study, no attempt was made to differentiate between CO₂ and H⁺ as the stimulus modality. Although not measured, seawater pH was undoubtedly reduced during hypercarbia ($P_{wCO_2}=6-6.5$ mmHg). Although this probably constitutes a significant reduction in pH, any specific impact of H⁺ on the cardiorespiratory responses is, however, unlikely. For example, we have demonstrated (J. E. McKendry and S. F. Perry; unpublished data) that bolus injections of sea water equilibrated with 2% (15 mmHg) or 4% (30 mmHg) CO₂ into the mouth of dogfish or Atlantic salmon (*Salmo salar*) (external injection) caused pronounced cardiorespiratory responses (increased f_V and V_{AMP} ; decreased f_H). These responses were not observed when aerated sea water was titrated to equivalent pH values (7.0 and 6.3, respectively) with HCl. Similarly, Reid et al. (Reid et al., 2000) demonstrated that the cardiorespiratory responses to hypercarbia in traira were not mimicked by external injection of HCl. Although these results suggest that changes in external pH, *per se*, do not trigger cardiorespiratory responses, they do not exclude a role for H⁺

in the net response to hypercarbia. Indeed, it is likely that the production of H⁺ within the chemoreceptor following the inward diffusion of CO₂ is a key mechanism underlying cardiorespiratory responses to hypercarbia.

Cardiorespiratory responses to hypercarbia in denervated dogfish

Denervation of all gill arches (*via* transection of all branchial branches of cranial nerves IX and X) eliminated all cardiorespiratory responses to hypercarbia. These results clearly demonstrate the existence of branchial chemoreceptors in dogfish that are stimulated by elevated CO₂ and/or H⁺ as well as providing evidence for the absence of central CO₂/H⁺ chemoreceptors. Whether the receptors are oriented externally and monitor inflowing water and/or are oriented internally and monitor the blood cannot be distinguished from the present experimental design. However, we (J. E. McKendry and S. F. Perry, unpublished data) have recently demonstrated that injections into the caudal vein of dogfish of saline equilibrated with 4% CO₂ ($P_{CO_2}=30$ mmHg) did not elicit any cardiorespiratory responses (unlike external injections of sea water gassed with 4% CO₂; see above). These data argue in favour of exclusively externally oriented CO₂ chemoreceptors.

Because all the gill arches were denervated in the present experiments, we cannot comment on the relative distribution of the branchial CO₂ receptors in dogfish. However, if distributed in a manner similar to O₂ chemoreceptors in elasmobranchs, they are likely to be diffusely distributed amongst all gill arches. However, unlike the additional location of O₂ chemoreceptors within the buccal cavity of dogfish (Butler et al., 1977), the CO₂ chemoreceptors appear to be confined to the gills (*i.e.* all cardiorespiratory responses were abolished despite continuing innervation of the orobranchial cavity *via* cranial nerves V and VII).

Because of the scarce and heterogeneous nature of existing data on cardiorespiratory responses to hypercarbia in fish, it is not yet possible to formulate a general model. For example, in tambaqui, the receptors mediating reflex hypercarbic bradycardia are localised to the first gill arch (Sundin et al., 2000), whereas in traira the receptors mediating the bradycardia are found within all the gill arches (Reid et al., 2000). Clearly, further studies on a variety of species are required to determine whether the first gill arch (as appears to be the case for O₂ chemoreception in most teleosts) is the predominant site of CO₂ chemoreception.

In other vertebrates, peripheral chemoreceptors are responsive to both O₂ and CO₂ (Iturriaga, 1993). In fish, however, it is unclear whether there is a single population of branchial receptors that are responsive to lowered O₂ and elevated CO₂ or two distinct populations of CO₂ and O₂ chemoreceptors. In dogfish, there is evidence for and against a single population of receptors. In support of a single receptor population, the cardiorespiratory responses to hypercarbia (increased f_V and V_{AMP} ; decreased f_H and P_a) are similar to the responses observed during hypoxia. In opposition, and perhaps

more importantly, external injections of cyanide (a potent activator of O₂ chemoreceptors) elicited bradycardia and hypotension but did not elicit the marked reduction in cardiac output that accompanies hypercarbia.

Cardiorespiratory responses to hypercarbia in atropinized dogfish

The marked attenuation of the hypercarbic bradycardia and ensuing reduction in cardiac output and blood pressure in atropinized fish was not surprising because increased vagal tone is the key contributor to reflex bradycardia in dogfish (Butler and Metcalfe, 1988). However, because the reflex bradycardia was not totally abolished by atropine, elevated blood P_{CO₂}, in itself, may contribute slightly to the hypercarbic bradycardia. The more interesting result was the blockade of the hyperventilatory responses during hypercarbia in the atropine-treated fish. Previous studies have demonstrated that O₂ chemoreception in fish (Burlleson and Milsom, 1995a) and mammals (Fitzgerald, 2000; Fitzgerald et al., 2000) involves an important cholinergic element. According to the 'cholinergic hypothesis' of O₂ chemotransduction for the mammalian carotid body (for a review, see Fitzgerald, 2000), acetylcholine is released from the O₂-sensing glomus cells and interacts with postsynaptic cholinergic receptors on neighbouring afferent neurons as well as with autoreceptors on the glomus cells themselves. There is evidence for the presence of both nicotinic and muscarinic receptors at both sites (Fitzgerald, 2000; Fitzgerald et al., 2000; Shirahata et al., 2000), although the relative contributions of these receptor subtypes to the overall chemotransduction process are unclear. It is generally accepted, however (see Fitzgerald, 2000), that the nicotinic and M₁ muscarinic receptors are excitatory, while the M₂ muscarinic receptors are inhibitory.

The absence of any ventilatory response to hypercarbia after atropine treatment suggests a critical involvement of muscarinic receptors in the chemosensory response of dogfish to CO₂. This finding is consistent with the results of earlier studies demonstrating that atropine blocks acetylcholine- or cyanide-evoked increases in trout gill O₂ chemoreceptor activity *in situ* (Burlleson and Milsom, 1995a) and that injection of muscarine markedly stimulates breathing in trout *in vivo* (Burlleson and Milsom, 1995b). Although the focus of the present study was on CO₂ rather than O₂ chemoreception, the observation that atropine eliminated the hyperventilatory responses to external cyanide suggests a crucial role for muscarinic receptors in the response of dogfish to both hypercarbia and hypoxia.

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References

- Axelsson, M. and Fritsche, R.** (1994). Cannulation techniques. In *Analytical Techniques* (ed. P. W. Hochachka and T. P. Mommsen), pp. 17–36. Amsterdam: Elsevier.
- Burlleson, M. L. and Milsom, W. K.** (1995a). Cardio-ventilatory control in rainbow trout. I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. *Respir. Physiol.* **100**, 231–238.
- Burlleson, M. L. and Milsom, W. K.** (1995b). Cardio-ventilatory control in rainbow trout. II. Reflex effects of exogenous neurochemicals. *Respir. Physiol.* **101**, 289–299.
- Burlleson, M. L. and Smatresk, N. J.** (2000). Branchial chemoreceptors mediate ventilatory responses to hypercapnic acidosis in channel catfish. *Comp. Biochem. Physiol. A* **125**, 403–414.
- Burlleson, M. L., Smatresk, N. J. and Milsom, W. K.** (1992). Afferent inputs associated with cardioventilatory control in fish. In *The Cardiovascular System* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 389–423. San Diego: Academic Press.
- Butler, P. J. and Metcalfe, J. D.** (1988). Cardiovascular and respiratory systems. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 1–47. Berlin, Heidelberg, New York, London, Paris, Tokyo: Springer-Verlag.
- Butler, P. J. and Taylor, E. W.** (1971). Response of the dogfish (*Scyliorhinus canicula* L.) to slowly induced and rapidly induced hypoxia. *Comp. Biochem. Physiol. A* **39**, 307–323.
- Butler, P. J., Taylor, E. W. and Short, S.** (1977). The effect of sectioning cranial nerves V, VII, IX and X on the cardiac response of the dogfish *Scyliorhinus canicula* to environmental hypoxia. *J. Exp. Biol.* **69**, 233–245.
- Fitzgerald, R. S.** (2000). Oxygen and carotid body chemotransduction: the cholinergic hypothesis – a brief history and new evaluation. *Respir. Physiol.* **120**, 89–104.
- Fitzgerald, R. S., Shirahata, M. and Wang, H. Y.** (2000). Acetylcholine is released from *in vitro* cat carotid bodies during hypoxic stimulation. *Adv. Exp. Med. Biol.* **475**, 485–494.
- Fritsche, R.** (1990). Effects of hypoxia on blood pressure and heart rate in three marine teleosts. *Fish Physiol. Biochem.* **8**, 85–92.
- Fritsche, R. and Nilsson, S.** (1993). Cardiovascular and ventilatory control during hypoxia. In *Fish Ecophysiology* (ed. J. C. Rankin and F. B. Jensen), pp. 180–206. London: Chapman & Hall.
- Graham, M. S., Turner, J. D. and Wood, C. M.** (1990). Control of ventilation in the hypercapnic skate, *Raja ocellata*. I. Blood and extraradial fluid chemistry. *Respir. Physiol.* **80**, 259–277.
- Heisler, N., Toews, D. P. and Holeyton, G. F.** (1988). Regulation of ventilation and acid–base status in the elasmobranch *Scyliorhinus stellaris* during hyperoxia induced hypercapnia. *Respir. Physiol.* **71**, 227–246.
- Iturriaga, R.** (1993). Carotid body chemoreception: the importance of CO₂, HCO₃⁻ and carbonic anhydrase. *Biol. Res.* **26**, 319–329.
- Kent, B. and Peirce, E. C. I.** (1978). Cardiovascular responses to changes in blood gases in dogfish shark, *Squalus acanthias*. *Comp. Biochem. Physiol.* **60C**, 37–44.
- Kinkead, R. and Perry, S. F.** (1991). The effects of catecholamines on ventilation in rainbow trout during external hypoxia or hypercapnia. *Respir. Physiol.* **84**, 77–92.
- McKendry, J. E. and Perry, S. F.** (2001). Cardiovascular effects of hypercapnia in rainbow trout (*Oncorhynchus mykiss*): a role for externally oriented chemoreceptors. *J. Exp. Biol.* **204**, 115–125.
- McKenzie, D. J., Burlleson, M. L. and Randall, D. J.** (1991). The effects of branchial denervation and pseudobranch ablation on

- cardioventilatory control in an air-breathing fish. *J. Exp. Biol.* **161**, 347–365.
- Milsom, W. K.** (1995a). Regulation of respiration in lower vertebrates: Role of CO₂/pH chemoreceptors. In *Advances in Comparative Physiology*, vol. 21, *Mechanisms of Systemic Regulation: Acid–Base Regulation, Ion Transfer and Metabolism* (ed. N. Heisler), pp. 62–104. Berlin: Springer-Verlag.
- Milsom, W. K.** (1995b). The role of CO₂/pH chemoreceptors in ventilatory control. *Braz. J. Med. Biol. Res.* **28**, 1147–1160.
- Milsom, W. K., Sundin, L., Reid, S., Kalinin, A. and Rantin, F. T.** (1999). Chemoreceptor control of cardiovascular reflexes. In *Biology of Tropical Fishes* (ed. A. L. Val and V. M. F. Almeida-Val), pp. 363–374. Manaus: INPA.
- Olson, K. R., Conklin, D. J., Farrell, A. P., Keen, J., Takei, Y., Weaver, L., Smith, M. P. and Zhang, Y.** (1997). Effects of natriuretic peptides and nitroprusside on venous function in trout. *Am. J. Physiol.* **273**, R527–R539.
- Perry, S. F., Fritsche, R., Hoagland, T., Duff, D. W. and Olson, K. R.** (1999a). The control of blood pressure during external hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **202**, 2177–2190.
- Perry, S. F. and Gilmour, K. M.** (1996). Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (*Squalus acanthias*) and a teleost (*Oncorhynchus mykiss*). *J. Exp. Biol.* **199**, 2105–2118.
- Perry, S. F., Gilmour, K. M., Bernier, N. J. and Wood, C. M.** (1999b). Does gill boundary layer carbonic anhydrase contribute to carbon dioxide excretion: a comparison between dogfish (*Squalus acanthias*) and rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **202**, 749–756.
- Perry, S. F. and Wood, C. M.** (1989). Control and coordination of gas transfer in fishes. *Can. J. Zool.* **67**, 2961–2970.
- Randall, D. J.** (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.* **100**, 275–288.
- Randall, D. J., Heisler, N. and Drees, F.** (1976). Ventilatory response to hypercapnia in the larger spotted dogfish *Scyliorhinus stellaris*. *Am. J. Physiol.* **230**, 590–594.
- Reid, S. G., Sundin, L., Kalinin, A. L., Rantin, F. T. and Milsom, W. K.** (2000). Cardiovascular and respiratory reflexes in the tropical fish, traíra (*Hoplias malabaricus*): CO₂/pH chemoresponses. *Respir. Physiol.* **120**, 47–59.
- Satchell, G. H.** (1961). The response of the dogfish to anoxia. *J. Exp. Biol.* **38**, 531–543.
- Shelton, G., Jones, D. R. and Milsom, W. K.** (1986). Control of breathing in ectothermic vertebrates. In *Handbook of Physiology*, section 3, *The Respiratory System*, vol. 2, *Control of Breathing* (ed. N. S. Cherniak and J. G. Widdicombe), pp. 857–909. Bethesda: American Physiological Society.
- Shirahata, M., Ishizawa, Y., Rudisill, M., Sham, J. S., Schofield, B. and Fitzgerald, R. S.** (2000). Acetylcholine sensitivity of cat petrosal ganglion neurons. *Adv. Exp. Med. Biol.* **475**, 377–387.
- Short, S., Taylor, E. W. and Butler, P. J.** (1979). The effectiveness of oxygen transfer during normoxia and hypoxia in the dogfish (*Scyliorhinus canicula* L.) before and after cardiac vagotomy. *J. Comp. Physiol. B* **132**, 289–295.
- Smatresk, N. J.** (1990). Chemoreceptor modulation of endogenous respiratory rhythms in vertebrates. *Am. J. Physiol.* **259**, R887–R897.
- Smith, F. M. and Jones, D. R.** (1982). The effect of changes in blood oxygen carrying capacity on ventilation volume in the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **97**, 325–334.
- Sundin, L., Reid, S. G., Rantin, F. T. and Milsom, W. K.** (2000). Branchial receptors and cardiorespiratory reflexes in the neotropical fish, the tambaqui (*Colossoma macropomum*). *J. Exp. Biol.* **203**, 1225–1239.
- Taylor, E. W.** (1992). Nervous control of the heart and cardiorespiratory interactions. In *The Cardiovascular System* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 343–387. San Diego: Academic Press.
- Thomas, S.** (1983). Changes in blood acid–base balance in trout (*Salmo gairdneri* Richardson) following exposure to combined hypoxia and hypercapnia. *J. Comp. Physiol. B* **152**, 53–57.
- Thomas, S.** (1994). Extracorporeal circulation. In *Biochemistry and Molecular Biology of Fishes* (ed. P. W. Hochachka and T. P. Mommsen), pp. 161–167. Amsterdam: Elsevier.